Sex-reversal of Fall-Run Chinook salmon

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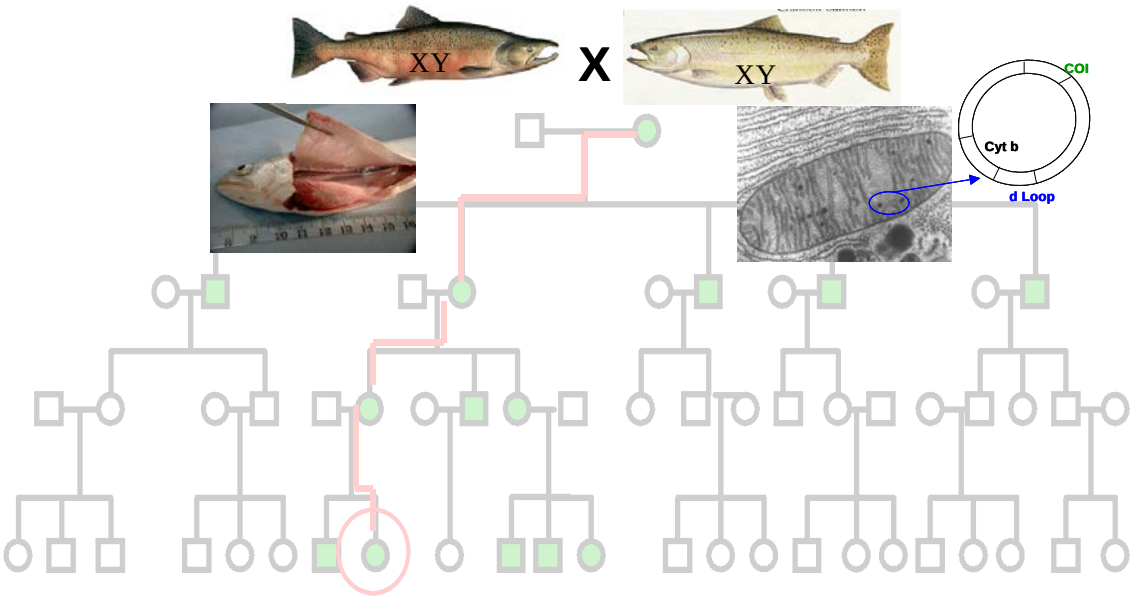

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Tricia Parker
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Red Bluff Fish and Wildlife Office
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RE: Final report for extension of cooperative agreement (#113322J006) 'Sex-reversal of Fall-Run Chinook salmon'

Tricia,

Outlined below are the accomplishments for each of the stated tasks and goals for the extension project to investigate 'apparent' sex-reversal of fall- and spring-run Chinook salmon in the Central Valley of California. I have also included a brief summary of the overall project objectives, reasons behind the impetus for our research, and implications of our observations that are pertinent to each of the stated tasks. If you have any questions regarding our final report, please do not hesitate to contact me.

Tasks

Task 1 focused on comparing sexual genotype and phenotype in adult specimens of fall- and spring-run Chinook salmon collected during spawned carcass surveys carried out on tributaries throughout the Sacramento River and San Joaquin River drainages. This information and statistical data analysis, specifically the spatial and temporal variability in distribution of 'apparent' XY females (phenotypic females with a male genotype) have been completed and are presented in the report following this cover letter.

Connection of task 1 with overall project

Strategic goals of the California Bay-Delta Authority (CBDA) involve the assessment of both at-risk species and water and sediment quality within the Central Valley. The main objective behind the assessment of water and sediment quality is to improve and/or maintain water and sediment quality conditions of toxic contaminants in all aquatic environments in the Bay-Delta estuary and watersheds to levels that do not adversely affect aquatic organisms, wildlife, and human health.

Due to characteristic life history traits, salmonids are particularly susceptible to the effects of exogenous sex steroids and xenoendocrine disrupting compounds in the environment. While there is evidence that sex determination is chromosomally based (Thorgaard and Gall 1979), there is a period during early development when the genetic mechanisms of sex determination may be labile to exogenous compounds. Many of the compounds that have been identified as xenoendocrine disruptors/sex steroid mimics are

hydrophobic and lipophilic, properties conducive to bioaccumulation in aquatic organisms. According to Piferrer and Donaldson (1994) the alevin yolk sac may act as a reservoir when these substances are exogenously encountered. Hunter et al. (1986) have shown that male chinook salmon are highly susceptible to sex reversal when exposed to exogenous estrogen from around the time of hatching to beyond the time when these fish begin to feed. The predisposition of the alevin yolk sac to absorb lipophilic substances and the fact that during the first week of the alevin stage in coho and chinook salmon, germ cells undergo a high rate of mitotic proliferation in the developing gonad (Piferrer and Donaldson 1993), exacerbates the sensitivity of sexual differentiation (i.e. - decision to develop as either a male or female) and gonad development to external factors. Environmental exposure of developing fish embryos to steroid hormones and endocrine disrupting compounds typically manifests as altered sexual development (i.e. – incomplete formation of a functional gonad) rather than altered sexual differentiation (reviewed in Tyler et al. 1998).

In this and previous studies (Williamson and May 2002, 2005a, *in press*) no examples of incompletely altered sexual development, such as intersex gonads (contain both ovarian and testicular tissue), have been observed in Central Valley Chinook salmon. Altered gonad development has not been observed during the 1999 (Williamson and May 2002), or 2002 and 2005 fall- and spring-run, respectively, adult Chinook carcass surveys conducted throughout the Central Valley of California (Williamson and May *in press*). These observations suggest that altered sexual development of gross gonad morphology, potentially owing to exposure to endocrine disrupting compounds (EDCs), is not a stressor to population persistence of Chinook salmon in the Central Valley. Furthermore, the lack of detectable variation in the proportion of 'apparent' XY-female fall (spatial or temporal) and spring (spatial) Chinook suggests that environmental influences on sexual differentiation are not a likely cause of incongruent genotypic and phenotypic sex in Chinook salmon in California's Central Valley.

It is important to note that these studies have not examined the potential of EDCs to elicit changes in the expression of genes directing the differentiation or development of gonads, influence the ability or timing of endocrine regulation of reproductive processes (i.e.- correct development of gametes within gonads), or result in sub-lethal effects (i.e. – suppression of immune system) in this species. This is an important distinction since exposure of developing Chinook embryos to EDCs may have consequences for population persistence that the present study was not intended to specifically address.

Task 2 was expanded to include an examination of the incidence of 'apparent' XY-female fish in three threatened spring-run Chinook salmon populations located in Butte, Deer, and Mill Creeks in the Sacramento R. drainage system. This information and statistical data analysis have been completed and are presented in the following report.

Connection of task 2 with overall project

The original objective of task 2 was to determine if sex-reversed male (XY-female = phenotypic female with a male genotype) fall-run Chinook salmon have a spatially heterogeneous distribution (i.e.- more likely to occur in one particular river basin or tributary compared to another) within the Central Valley. Observed spatial variation in the incidence of XY-females may suggest that an environmental component is responsible for producing these fish. This sort of information may be combined with data from other disciplines (landscape ecology, hydrology, geographic information technology) to survey for aquatic ecosystem pollutants from potential agricultural, livestock, and urban sources. Williamson and May (2002) described incongruent genotypic and phenotypic sex in fall-run Chinook collected during the 1999 carcass surveys throughout the Central Valley. Genetic sex was assessed using a single molecular probe (OtY1; Devlin et al. 1991, 1994). Initial results suggested that fall-run Chinook populations on tributaries that contained a salmon hatchery had a higher incidence of XY-females. In the subsequent study (presented below) we performed a more rigorous analysis of adult fall- and spring-run fish returning in 2002 and 2005, respectively. Larger sample sizes of fall-run fish were collected along the tributaries of the Sacramento and San Joaquin Rivers and collections were expanded into threatened spring-run populations. Furthermore, a second genetic marker for sex (Growth Hormone Pseudogene; Du et al. 1993) was utilized to corroborate data obtained from the first marker.

The impetus for expanding analysis to spring-run Chinook stemmed from the observation of six (17%) 'XY-females' out of 35 phenotypic females in a sample of 1999 Deer Creek fish (Williamson and May, *unpublished*). Moderate genetic diversity between the fall- and spring-run Chinook salmon populations in the Central Valley has been documented by Banks et al. (2000). Hybridization with fall-run Chinook is a major concern regarding the preservation of genetic diversity, persistence, and life history integrity of spring-run populations listed as threatened under the Endangered Species Act (Meyers et al. 1998). On tributaries where both fall- and spring-run Chinook salmon return to spawn overlap in the timing of their spawning migrations can potentially lead to interbreeding (CDFG-NMFS 2001). The presence of 'apparent' XY-females in the Deer Creek population suggests that (1)interbreeding between fall- and spring-run fish has occurred, (2) it has arisen independently in both runs, or (3) the origin of this mutation occurred before the differentiation of Fall and Spring runs.

The objective in expanding the second task to include spring-run fish was to determine if the three remaining spring-run populations on Butte, Deer, and Mill Creek experienced introgression with 'apparent' XY-females from the fall-run. Incongruent genotypic and phenotypic sex was compared between spring-run Chinook salmon populations. Due to

experimental data and further reflection upon the results of earlier controlled breeding experiments, we decided to focus our effort on the comparison of mtDNA between the two runs. This decision was based on the observation that half of the female offspring produced by crosses of 'apparent' XY-females with normal males had incongruent genotypic and phenotypic sex (Williamson and May 2005a). This observation formed the basis of the proposed hypothesis to examine mtDNA diversity within the Fall-Run (i.e.- H_o-4: The diversity of mtDNA haplotypes for genetically normal and XY-female Fall-Run Chinook salmon sampled throughout the Central Valley is the same.). Since the genetic change, mutant gene or chromosomal rearrangement, inherited by half of the female offspring is also inherited by half of the male offspring in families produced by XY-females, transfer of the genetic change to another mtDNA haplotype lineage is mediated by those male offspring carriers that successfully reproduce. Hence, it is highly unlikely that only a single mtDNA haplotype occurs in "apparent" XY-female Fall Chinook. In this respect, it is infeasible to infer hybridization between the fall- and spring-run populations by attempting to correlate a particular mitochondrial haplotype with 'apparent' XY-female fish. Despite this fact, other hypotheses (presented below) could still be tested. Continued evaluation of the data, where possible, has provided useful information regarding the time frame over which 'apparent' XY-females have likely been present in Central Valley Chinook salmon.

Task 3 was expanded to include (Task #3-1) examining the mitochondrial (mtDNA) haplotypes of the two different classes of female Chinook (normal and sex-reversed) and (Task #3-2) examining the DNA sequences of these male markers for differences. The results (description and statistical analysis) of mtDNA haplotype distributions in fall- and spring-run Chinook have been completed and are presented in the report following this cover letter.

Connection of task 3 with overall project

The original focus of task 3 was to perform experimental crosses between 'apparent' XY-female and normal male fall-run Chinook salmon. Offspring genotypic and phenotypic sex ratios were compared within individual families produced by 'apparent' XY-females, and compared with those ratios in offspring produced by 'control' crosses between normal males and females. Performing the crosses served two purposes. First, it allowed validation of Mendelian inheritance of the OtY1 marker in both normal and sex-reversed scenarios. Second, it provided evidence of whether or not gametes from sex-reversed individuals were capable of being fertilized and producing viable offspring. The results and analysis of sex marker inheritance in the crosses has been presented in Williamson and May (2005a).

Breeding experiment results suggested two alternative models to explain the observations of phenotypic female offspring with incongruent sexual genotype produced by XY-female fall-run Chinook. The first model describes a mutational event wherein both Y-chromosome specific markers are translocated from the Y- to the X-chromosome. The second model suggests a variety of mutational events that may have resulted in the

inactivation of the sex-determining region. In order to ascertain the nature of the genetic change that results in XY-female Chinook we had proposed to perform DNA sequencing outward along the chromosome from the Y-chromosome specific markers (OtY1 and Growth Hormone pseudogene) in both 'apparent' XY-female and genetically normal, male Fall-Run Chinook. However, discussion with colleagues has indicated that this approach has been unsuccessful.

Analysis of DNA Sequence along Y-chromosome of Chinook salmon (Task #3-2) has already been attempted by Dr. Rob Devlin (Department of Fisheries and Oceans, Canada). Devlin has already performed extensive DNA sequencing of the interstitial regions adjacent to OtY8 tandem repeats on the Chinook Y chromosome. Only highly repetitive DNA sequences were encountered in these regions, therefore, little useful Y chromosome specific information was obtained. Furthermore, the highly repetitive DNA sequences in the area of the Y chromosome examined precludes the ability to perform a "chromosome walk" (DNA sequencing) along the length of the chromosome. Because of the high repetition of DNA sequences in the region where OtY8 (contains OtY1) is located, it is difficult to ascertain the order of DNA sequences relative to one another or to their location on the Y chromosome. Furthermore, despite the fact that the 'apparent' XY-females carry only ~ 25% of the OtY8/GH-psi signal of normal males, that signal is likely due to a very high copy number of these sequences. These markers may span several 100 thousand base pairs of DNA sequence. The inability to unambiguously "walk" outward from OtY8 in one specific direction or another along the chromosome of 'apparent' XY-females prevents us from pursuing this line of research we had originally posed as a part of Task 3 in the extension. All other tasks have been completed as proposed.

Goals

Communicate research results to scientific and fisheries management communities – Oral presentation of project results was given at national and regional scientific conferences, and to the collaborators of a separate, complementary investigation of 'apparent' XY-female fall-run fish. These presentations included:

Civil and Environmental Engineering Dept., University of California, Berkeley, CA, 05/07- Title: Genetic analysis of 'apparent' XY-female fall Chinook salmon in California.

4th Biennial CALFED Bay-Delta Program Science Conference, Sacramento, CA, 10/06- Title: Chromosomal rearrangements in 'apparent' sex-reversed fall Chinook salmon in California.

136th American Fisheries Society Annual Meeting, Lake Placid, NY, 09/06- Title: Cytogenetic analysis of 'apparent' XY-female Fall Chinook in California.

Coast-wide Salmonid Genetics Meeting, Santa Cruz, CA, 06/06- Title: Cytogenetic analysis of 'apparent' XY-female Fall Chinook in California.

US Fish and Wildlife Service, Habitat Restoration Committee, Red Bluff, CA, 08/05- Presented research results and future research goals regarding 'apparent' sex-reversal of Fall Chinook in California's Central Valley to Jr. and Sr. Biologists (USFWS). Title: Sex-reversal in Central Valley Fall Chinook: altered sexual differentiation or mutation?

Publish research results in peer-reviewed literature – Our research results have been accepted for publication in the journal Transactions of the American Fisheries Society and may be referenced as follows:

Williamson, Kevin S., and Bernie May. Mitochondrial DNA haplotype diversity in apparent XY-female Fall- and Spring-Run Chinook salmon in California's Central Valley. Transactions of the American Fisheries Society. (*in press*).

Thank you,

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Mitochondrial DNA haplotype diversity in 'apparent' XY-female fall- and spring-Run Chinook Salmon in California's Central Valley

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Abstract

Mitochondrial DNA (mtDNA) haplotype diversity between 'apparent' XY-female and genetically normal females in California's Central Valley fall- and spring-run Chinook salmon were compared in order to ascertain whether or not a sub-set of mtDNA haplotypes are unique to 'apparent' XY-female Chinook. Two Y chromosome markers, OtY1 and Growth Hormone Pseudogene (GH-Ψ), were used to screen spring Chinook salmon collected on Butte, Deer, and Mill Creeks in 2005 for the presence of 'apparent' XY-females. Fall-run fish collected from 2002 to 2004 had previously been screened for genotypic sex using the same Y markers. A 237 bp region of the mitochondrial d-loop segment was sequenced to determine mtDNA haplotypes of XY-female and randomly selected normal female fall- and spring-run fish. Apparent XY-females, according to OtY1 and GH-Ψ, were observed in all three newly sampled spring-run populations at 26%, 33%, and 2%, respectively. No significant differences in haplotype distributions between normal and 'apparent' XY-females suggest that these fish are equally represented within the separate fall- and spring-runs. It is possible that 'apparent' XY-female Chinook salmon have been present in the Central Valley of California prior to the genetic divergence of the fall- and spring-runs. Furthermore, 'apparent' XY-female Chinook may have become established in this region through a founder event that resulted in fewer modes of variation in genotypic and phenotypic sex compared to populations in the interior Columbia River Basin.

Introduction

Reports of phenotypic female Chinook salmon (*Oncorhynchus tshawytscha*) that have a male genotype raised concerns of altered sexual differentiation owing to environmental exposure to endocrine disrupting compounds in the Pacific Northwest (Nagler et al. 2001) and in California (Williamson and May 2002). When Chinook salmon genomic DNA is probed with sex-specific chromosome markers, OtY1 (Devlin et al. 1994) and Growth Hormone pseudogene (Du et al. 1993), males and females exhibit differential signals. The basis of the differential signal of these markers depends upon the presence of a Y chromosome in the individual tested. However, recent evidence for Chinook salmon in the Pacific Northwest (Chowen and Nagler 2004a,b) and in California (Williamson and May 2005a) indicates that observed incongruence between genetic and phenotypic sex has a genetic basis rather than altered sexual differentiation owing to endocrine disruption. Controlled breeding experiments (Williamson and May 2005a) have provided supporting evidence that a genetic rearrangement or mutation, transmitted to half of both male and female offspring, is responsible for the 'apparent' sex-reversed male (XY-female) fall-run Chinook salmon in the Central Valley. Nagler et al. (2001) suggested that the Y-chromosome arm containing the OtY1 sequence may have translocated to the X chromosome or an autosome. Another possibility is that the observed incongruence between sexual genotype and phenotype is evidence of past genetic rearrangement involving some or all of the several hundred copies of OtY1 (Devlin et al. 1998) between the Y and the X chromosome/autosome (Chowden and Nagler 2004b). Williamson and May (2005a) have suggested that a mutation affecting either expression or regulation of the primary sex-determining locus (*SEX*) on the Y-chromosome may be a cause of 'apparent' XY-female fall Chinook in California. In any event, 'apparent' XY-female Chinook salmon appear to be a population specific phenomenon. The genetic rearrangement or mutation responsible for producing 'apparent' XY-female fall-run Chinook salmon in California has likely arisen independently of any such genetic changes that have occurred in other more northern populations of Chinook (Williamson and May 2005a). The examination of multiple sex markers within individuals showed a lesser degree of variation in California fall-run Chinook salmon (Williamson and May 2005a) compared to that of more northern populations (Devlin et al. 2005), suggesting the fall-run salmon experienced a single genetic change different from that of the northern populations. If the presence of 'apparent' XY-female Chinook within the Central Valley is due to a single, recent mutational event, then all such fish may not yet have spread to other lineages within the fall-run. The matrilineal inheritance of mitochondrial DNA (Gyllensten et al. 1985) may be exploited to evaluate whether or not 'apparent' XY-female Chinook spread from a single female into other mtDNA haplotype lineages. Five mtDNA haplotypes, previously described by Nielsen et al. (1994), occur at varying frequencies within Central Valley fall- and spring-run Chinook salmon. By performing DNA sequencing of the mtDNA d-loop segment in 'apparent' XY-female and randomly chosen, genetically normal female fall- and spring-run Chinook salmon we test the hypothesis of no difference in mitochondrial haplotype diversity between 'apparent' XY- and genetically normal females in both runs. The observation of only a subset of all possible mtDNA haplotypes in 'apparent' XY-female fall-run Central Valley Chinook would lend support to the idea that Chinook with incongruent sexual genotype and phenotype are the result of a recent

single, isolated mutational event that has not yet spread throughout the fall population as a whole.

Methods

Field sampling sites - Fin clips were taken and gross gonad morphology was observed for 659 phenotypic female and 281 male fall-run Chinook salmon during the 2002 adult carcass surveys conducted throughout the Central Valley. Survey sites for fall-run Chinook within the Sacramento River drainage system included the American River, Battle Creek, Coleman National Fish hatchery (on Battle Creek), Feather River, Feather River Hatchery, Nimbus Hatchery, and the Yuba River. Survey sites for fall-run fish within the San Joaquin River drainage system included the Merced River Hatchery, Merced River, Mokelumne River Hatchery, Stanislaus River, and Tuolumne River. Personnel from CDFG took fin-clips and observed gonad morphology for spring Chinook during the 2005 carcass surveys on Butte Creek, Deer Creek, and Mill Creek (N= 76, 61, and 26, respectively).

Genetic screening to detect 'apparent' XY female fish – Genomic DNA from fin-clips of phenotypic male and female fall- and spring-run Chinook salmon was extracted using a QIAgen DNeasy Blood and Tissue Kit (QIAgen, Valencia, California). The genotypic sex of individuals was determined by polymerase chain reaction (PCR) assays using both OtY1 (Devlin et al. 1994) and Growth Hormone pseudogene (Du et al. 1993), and the Y chromosome markers were scored as described in Williamson and May (2005a).

Statistical analysis of spatial and temporal variability in distribution of 'apparent' XY females – Spatial or temporal variability in the proportion of 'apparent' XY-female fish observed may be indicative of an environmental influence on sex determination in fall-run Chinook. The proportions of 'apparent' XY-female fall-run Chinook salmon sampled from the same locations during the 1999 (Williamson and May 2002) and 2002 (this study) adult carcass surveys were compared using the Wilcoxon Signed-Ranks test (Wilcoxon 1945) as implemented in the software program JMP-in version 4.0.3 (SAS Institute Inc., Cary, North Carolina). This was done to evaluate if there was a significant (α=0.05) increase or decrease in the proportion of 'apparent' XY-female Chinook salmon observed in the Sacramento or San Joaquin R. Basin between 1999 and 2002. Geographic variation (Sacramento vs. San Joaquin) of the proportion of 'apparent' XY-females observed in 1999 (Williamson and May 2002) and 2002 was also analyzed using the Wilcoxon Signed-Ranks test. This was done to evaluate if there were a significantly (α=0.05) higher proportion of 'apparent' XY-female Chinook salmon observed in one river system or another. Temporal variation of 'apparent' XY-female spring Chinook was not assessed due to lack of temporal sampling. Spatial variation of the proportion of 'apparent' XY-female spring observed between Butte, Deer, and Mill Creeks was assessed using Fishers Exact test (Fisher 1934) as implemented in JMP-in version 4.0.3 (SAS Institute Inc., Cary, North Carolina).

Mitochondrial DNA amplification and sequencing – Analysis of DNA sequence was performed on the mtDNA d-loop segment of all 'apparent' XY-females collected in 2002

as well as a randomly chosen subset of genetically normal females collected from each of the rivers sampled in 2002. Due to poor DNA quality, successful, unambiguous, bi-directional DNA sequencing was achieved for only a subset of the normal and XY-female spring-run (18 and 10, respectively) Chinook salmon. The mtDNA d-loop segment was amplified using 20 ng of genomic DNA, 5.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.8 μM of each PCR primer, and 0.75 Units of T_{aq} DNA Polymerase, 20 mM Tris (pH 8.5) and 50 mM KCl in 10 μl volumes. PCR assays were performed in a PTC100 thermal cycler (MJ Research, San Francisco, California) under the following conditions: one denaturation cycle at 95°C for 150 seconds, then 35 amplification cycles of 94°C for 40 s, 50°C for 60 s, and 72°C for 120 s. The two DNA primers (modified* from Nielsen and Gan 1994; Nielsen et al. 1994) used for PCR, S-phe2* (5'-AGGGTCCATATTAACAGCTTC-3') and P2 (5'-TGTTAAACCCCTAAACCAG-3'), amplify approximately 237 bp of the 3' end of the mtDNA d-loop and the first 34 nucleotides of the phenylalanine tRNA in fall- and spring-run Chinook salmon. The S-Phe2 primer was redesigned further downstream of the original in order to alleviate DNA sequencing ambiguities.

Electrophoresis of PCR products (3 μl) was performed on a 5% acrylamide 7.5 M urea gel (Biorad Inc., Sigma Inc., respectively) made with 110 mM Tris (pH 8.3), 90 mM Borate, 2.5 mM EDTA, and stained with Gel Star (Clare Chemical Research, Dolores, CO). Reference marks were made on the gel cassette which was then scanned using a Molecular Dynamics 595 fluorimager (Sunnyvale, California). A printout of the scanned gel image was placed under the cassette and oriented with the reference marks. A small acrylamide plug containing the amplified product was excised from the gel and transferred to a 1.5 ml eppendorf tube containing 100 μl of highly purified water. Two μl of this mixture was used as a template in a second PCR reaction using the same primers, reagents, and thermal cycler conditions to generate sufficient PCR product for DNA sequencing.

Prior to DNA sequencing unincorporated deoxynucleotide triphosphates from PCR amplification reactions were removed using QIAgen PCR clean up kit (QIAgen, Valencia, California) following the manufacture's instructions, and PCR product concentration was quantified using a Molecular Dynamics 595 fluorimager (Sunnyvale, California). Bidirectional sequencing reactions for the mtDNA control region were performed using either the P2 or S-phe2 primers separately in the BigDye® Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA), and removal of unincorporated dideoxynucleotide triphosphates from sequencing reactions was performed using a Clean SEQ™ Kit (Agencourt Bioscience Corporation) following the manufacture's instructions. The resulting product was directly sequenced by capillary gel electrophoresis on an ABI3730 DNA sequencer (Pop4 polymer, 80cm capillary).

Analysis of mitochondrial DNA haplotype distributions – DNA sequence electropherograms were visualized and aligned using Sequencher ver.4.5 (Gene Codes Corporation, Ann Arbor, MI). Positions of variable nucleotide sites were based according to the d-loop segment reference sequence (Genbank accession #AF 392054). Observed haplotypes were named according to the nomenclature used by Nielsen et al. (1994). Observed mtDNA haplotype frequency distributions for normal and 'apparent' XY-female fish were compared to one another in both the fall- and spring-run using a

Likelihood Ratio Chi-Squared test as implemented in JMP-in ver. 4.0.3 (SAS Institute Inc., Cary, North Carolina). Central Valley fall-run Chinook salmon constitute a single genetically homogeneous population both spatially and temporally (Williamson and May 2005b). This suggested that it was not inappropriate to pool either normal, or 'apparent' XY-females sampled throughout the Central Valley.

Results

Out of 659 phenotypic female fall Chinook examined in 2002, 26 out of 392 (7%) and 32 out of 226 (~14%) from the Sacramento and San Joaquin River systems, respectively, were classified as genetic males according to both molecular markers (Table 1). The remaining 601 (91%) phenotypic females were classified as genotypic females and all 281 phenotypic males were classified as genotypic males according to both Y chromosome markers. Both Y markers were successfully PCR amplified in only a small number of the Butte, Deer, and Mill Creek spring-run Chinook phenotypic females (21, 24, and 14, respectively). "Apparent' XY-females were observed in the Butte, Deer, and Mill Creek spring-run Chinook populations (6, 8, and 2, respectively; Table 1). Phenotypic male spring Chinook were genotyped as male fish according to both Y markers in the Butte, Deer, and Mill Creek populations (25, 33 and 12, respectively).

No significant temporal variation in the proportion of 'apparent' XY-female fall Chinook salmon was detected in either the Sacramento R. or San Joaquin R. drainages (Signed rank = -8.5, p = 0.09, and Signed rank = 0.5, p = 1.00, respectively). Likewise, no significant geographic variation in the proportion of 'apparent' XY-female fall Chinook was detected between the two river basins (Signed rank = -1.5, p = 0.500). No significant spatial variation in the proportion of 'apparent' XY-female spring-run Chinook was detected between any of the three sampling sites (Butte vs. Deer Creeks, Butte vs. Mill Creeks, and Deer vs. Mill Creeks, two-tailed P = 1.00, 0.69, and 0.46, respectively).

Sequencing of mtDNA haplotypes was performed for normal and XY-female fall- (75 and 58, respectively) and spring-run (18 and 10, respectively) Chinook salmon. Five mtDNA haplotypes were observed in normal and 'apparent' XY-female fall Chinook (Table 2). Comparison of observed haplotype frequency distributions for normal and 'apparent' XY-female fall and spring Chinook indicated no significant differences. (Likelihood ratio χ^2=5.57, df=4, p=0.23, and χ^2=0.95, df=3, p=0.918, respectively). The average cell counts are less than five in the Likelihood Ratio Chi-Squared test for the spring Chinook mtDNA haplotype distribution. Accordingly the results may be suspect.

Discussion

The mtDNA haplotype data suggest that the mtDNA diversity of 'apparent' XY-female Chinook salmon is at equilibrium with that of normal females within the separate fall- and spring-runs. All mtDNA haplotypes previously observed in fall- and spring-run Chinook (Nielsen et al. 1994) were also observed in 'apparent' XY-female fall Chinook sampled from 2002-2004 and spring Chinook sampled in 2005 (Table 2). Nielsen et al.

(1994) did not observe mtDNA haplotype CH3 in spring-run Chinook salmon. In this study, however, spring-run 'apparent' XY-females with mtDNA haplotype CH3 (Table 2) were observed in each population examined. MtDNA Haplotype CH3 was likely not observed by Nielsen et al. (1994) due to the small sample size (N=15) for the single population they examined. The relative percentages of spring Chinook 'apparent' XY-females observed were comparable in magnitude to those observed for the fall-run on other tributaries (Table 1). In addition, no significant differences were observed in the haplotype distributions between normal and 'apparent' XY-female fish in either the fall- or spring-run. Females, as would be the case with the 'apparent' XY-females, transmit their mitochondria to each of their offspring. Both the phenotypic female offspring and their genetically normal female siblings produced by an 'apparent' XY-female would inherit the same mitochondrial haplotype. Similarly, half of the phenotypic male offspring would carry the mutation and both they and their genetically normal male siblings would have the same haplotype. If male carriers of the mutation successfully reproduced with a female with a different mtDNA haplotype, the males would pass the mutation onto another mitochondrial haplotype lineage as their 'affected' daughters from that pairing.

It is not known whether 'apparent' XY-females incur a reproductive fitness disadvantage thereby decreasing the probability that the genetic rearrangement or mutation carried by these fish is sustained within the population. While Williamson and May (2005a) did not rigorously test differences in reproductive success between normal and 'apparent' XY-female fall Chinook salmon, neither dissimilarity in gross gonad morphology of offspring, nor notable differences in offspring survival to the smolt stage have been observed. Since the sex chromosomes of many salmonids have not diverged to the point where vital genetic information is missing from the Y relative to the X chromosome (May et al. 1989; Allendorf et al. 1994; Devlin et al. 2001), it is possible (at least in Chinook) that by inheriting a genetic rearrangement or mutation involving one of the sex chromosomes would result in infertility, and thereby selecting against 'apparent' XY-females over time.

It is possible that 'apparent' XY-female Chinook have been present in the Central Valley of California long enough for the mtDNA diversity of normal and 'apparent' XY-females to reach equilibrium with one another in both the fall- and spring-runs. Banks et al. (2000) used microsatellites to reveal moderate genetic diversity between the fall- and spring-run Chinook salmon populations in the Central Valley. Similarly, Nielsen et al. (1994) observed different mtDNA haplotype frequency distributions between fall-and spring-run fish. Sufficient time has passed to allow fall- and spring-run Chinook salmon to have diverged genetically from one another. Given that the mtDNA haplotype frequency distributions for 'apparent' XY-females is not significantly different from that of normal females within each seasonal run, and that both runs have diverged genetically from one another, the genetic event responsible for producing 'apparent' XY females may have occurred before the two runs diverged.

The coincidental occurrence of Chinook salmon with incongruent genotypic and phenotypic sex in populations from the interior Columbian River Basin and the Central Valley of California may reflect a common event underlying this phenomenon in both groups of fish. Devlin et al. (2005) observed low to moderate proportions (0.3-19.4%) of phenotypic females with a male genotype at both Y markers in fall- and summer-run

Chinook salmon populations in the Columbia River Basin. Other variants observed in these populations included phenotypic males lacking both Y markers as well as individuals of either phenotypic sex having an incongruent sexual genotype at one or the other marker. Waples et al. (2004) examined allele frequency variation at 32 allozyme loci and found that Central Valley Chinook and Interior Columbia River Basin fall- and summer-run Chinook populations are minimally divergent (D. Teel, NOAA Fisheries, personal communication) from one another. The patterns of incongruent genotypic and phenotypic sex in and the minimal genetic divergence between Central Valley and Columbia River populations suggest that the genetic event responsible for producing 'apparent' XY-females may have originated in a progenitor that had expanded its distribution into both regions. In contrast, Williamson and May (2005a) suggested that the genetic change responsible for producing 'apparent' XY-females in California had likely arisen independently of any such changes that had occurred in more northerly populations. An alternative explanation may be that the far lesser degree of variation in California fall-run Chinook salmon (Williamson and May 2005a) compared to that of more northern populations (Devlin et al. 2005) may be due to a founder event as this species expanded its range southward.

A recent recombination event between the sex chromosomes and the subsequent movement of individuals carrying the genetic change is one alternative explanation for equal representation of normal and 'apparent' XY-females throughout the diversity of mtDNA haplotypes in the fall- and spring-run populations. Except for the region immediately adjacent to the sex-determining region the remainder of the pseudoautosomal sex chromosomes retains sufficient homology for recombination to occur (May et al. 1989; Allendorf et al. 1994). High copy number of (at least) the OtY1 marker on the Y (Devlin et al. 1998) coupled with the pseudoautosomal nature of the sex chromosomes raises the possibility of recombination involving Y chromosome markers. As suggested by Chowden and Nagler (2004b) selection of adults for gamete collection and egg fertilization involve a large degree of artificial manipulation, and therefore may lead to the non-random selection of genotypes. Regional dispersal of 'apparent' XY-female fish may have been inadvertently facilitated by historical interbasin transfers of eggs and fry between Central Valley hatcheries (CDFG-NMFS 2001), and/or straying of fish in CA rivers (Hallock and Reisenbichler 1979; Sholes and Hallock 1979). In either case it is possible for 'apparent' XY-female fish to have been spawned in a hatchery and the offspring moved to, or stray into and have been spawned in another hatchery. Hence, the recent origin of 'apparent' XY-females due to a recombination event and their subsequent spread throughout Central Valley Chinook populations cannot be overlooked.

It is possible that environmental sex determination may confound the ability to detect a founder event by introducing individuals with incongruent genotypic and phenotypic sex into a population. Sexual differentiation, and thus sex ratios, in fish can be influenced by environmental variables such as temperature (Bull 1983), pH (Rubin 1985) as well as exposure to steroid hormones and environmental pollutants. There is limited evidence that sex determination in Pacific salmonids is thermolabile. Craig et al. (1996) exposed developing eggs, obtained from pure and reciprocal hybrid crosses of anadromous and non-anadromous (kokanee) sockeye salmon (*O. nerka*), to fluctuating water temperatures. Relative to the sex ratios of control families maintained at a constant

temperature, families that experienced elevated temperature during embryonic development had strongly female-biased (62-84%) sex ratios. However, Nagler et al. (2003) observed no significant sex ratio differences in spring-run Chinook salmon due to daily temperature changes during the period of embryonic development, which is the time when the gonad develops sexually.

Chinook salmon and other fish treated with steroid hormones (Baker et al. 1988; Piferrer and Donaldson 1992; Devlin et al. 1994) or pollutants (Jobling et al. 1998; Larsson et al. 2000; Afonso et al. 2002) during early development may have an altered phenotypic sex (Rodgers-Gray et al. 2001) due to changes in organogenesis of the gonads or accessory sexual structures. For instance, hormone treatment or exposure to endocrine disrupting compounds (EDCs) may result in intersex gonads (contain both ovarian and testicular tissue) depending upon the timing of exposure and the developmental state of the gonad (reviewed in Tyler et al. 1998). Intersex gonads (Jobling et al. 1998) and disrupted development of gonadal ducts (Rodgers-Gray et al. 2001), possibly due to environmental EDC exposure, have been observed in natural populations of roach (*Rutilis rutilis*). However, to the best knowledge of the authors there have been no known documented cases of "endocrine disruption" of sexual differentiation in natural populations of salmonids. In this and previous studies (Williamson and May 2002, 2005a) no examples of incompletely altered sexual differentiation, such as intersex gonads, have been observed in Central Valley fall-run Chinook salmon. Furthermore, the lack of detectable variation in the proportion of 'apparent' XY-female fall (spatial or temporal) and spring (spatial) Chinook suggests that environmental influences on sexual differentiation are not a likely cause of incongruent genotypic and phenotypic sex in Chinook salmon in California's Central Valley.

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Table 1 – Distribution of 'apparent' XY-female fall-run (F) Chinook salmon collected during the 2002 carcass surveys in the Central Valley of California. All 281 phenotypic males collected during 2002 were genotyped as males (data not shown). Total number of phenotypic females screened for each site is in parentheses. *Spring-run (S) Chinook were sampled during 2005.

Sampling Location	Run	Phenotypic Females w/ male genotype by OtY1 and GH-Ψ	% XY-females
Sacramento R. Basin:			
American River	F	1 (29)	3
Battle Creek	F	12 (46)	26
Clear Creek	F	--	--
Coleman National Fish Hatchery	F	3 (185)	2
Feather R. Hatchery	F	2 (43)	5
Feather River	F	2 (30)	7
Nimbus Hatchery	F	5 (48)	10
Yuba River	F	1 (11)	9
*Butte Creek	S	6 (21)	29
*Deer Creek	S	8 (24)	33
*Mill Creek	S	2 (14)	14
Sacramento R. Basin Total:		**26 (392)**	**7**
San Joaquin R. Basin:			
Merced River	F	4 (39)	10
Merced River Hatchery	F	13 (50)	26
Mokelumne River	F	--	--
Mokelumne River Hatchery	F	5 (50)	10
Stanislaus River	F	4 (43)	9
Tuolumne River	F	6 (44)	14
San Joaquin R. Basin Total:		**32 (226)**	**14**

Table 2 – Observed mtDNA haplotypes in normal and XY-female fall- and spring-run Chinook salmon collected during the 2002 and 2005, respectively, spawning surveys. Haplotype percentages are given in parentheses. Mitochondrial haplotypes (CH1-5) are consistent with those presented in Nielsen et al. (1994).

Female		Observed mtDNA Haplotype				
Category	**N**	**CH1**	**CH2**	**CH3**	**CH4**	**CH5**
2002 Fall-Run						
Normal	75	20 (0.27)	8 (0.11)	10 (0.13)	16 (0.21)	21 (0.28)
XY	58	8 (0.14)	4 (0.07)	14 (0.24)	13 (0.22)	19 (0.33)
Total	133	34 (0.23)	13 (0.09)	25 (0.17)	31 (0.21)	43 (0.29)
Neilsen et al. 1994	138	46 (0.33)	5 (0.04)	17 (0.12)	40 (0.29)	30 (0.22)
2005 Spring-Run						
Normal	18	5 (0.28)	0	4 (0.22)	3 (0.17)	6 (0.33)
XY	10	3 (0.30)	0	3 (0.30)	2 (0.20)	2 (0.20)
Total	28	8 (0.29)	0	7 (0.25)	5 (0.18)	8 (0.29)
Neilsen et al. 1994	15	2 (0.13)	0	0	9 (0.60)	4 (0.27)